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Enterotoxigenicity of *Vibrio parahaemolyticus* with and without Genes Encoding Thermostable Direct Hemolysin

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Vibrio parahaemolyticus **produces a thermostable direct hemolysin (TDH) that has been implicated in the pathogenesis of diarrheal disease caused by this organism. However, previous studies attempting to demonstrate the contribution of the hemolysin to virulence have been inconclusive. We investigated this putative virulence factor by using an isogenic TDH-negative (TDH^-) strain constructed by specifically inactivating the two copies of the *tdh* gene encoding TDH. The enterotoxigenicities of the parent strain (AQ3815) and the mutant strain were tested by adding sterile culture supernatants to rabbit ileal tissue mounted in Ussing chambers. The culture filtrate of the parent strain produced a significant increase in short-circuit current (Isc), compared with the change induced by the TDH^- mutant. The capacity of the culture filtrate of AQ3815 to increase the Isc was reduced by neutralization with anti-TDH serum, and the return of the cloned *tdh* gene to the TDH^- mutant restored the ability to increase the Isc. These results were corroborated by rabbit ileal loop assays in which AQ3815 caused fluid accumulation but the TDH^- mutant did not. No microscopic damage was seen in mucosal tissues exposed to the culture filtrate of either strain. These results indicate that TDH has an enterotoxigenic effect on rabbit small intestine and could be responsible for the watery diarrhea seen with *V. parahaemolyticus*.**

Vibrio parahaemolyticus is a marine bacterium that can cause gastroenteritis, the major clinical symptoms being diarrhea, abdominal cramps, nausea, and vomiting (1, 17, 19). Although the pathogenic mechanisms of this organism are not well understood, proposed virulence factors or properties include thermostable direct hemolysin (TDH), other extracellular or cell-associated toxins or enzymes (3, 14, 16, 17, 19, 21, 34, 36, 38, 39), adherence (13, 17, 19, 20), and invasiveness (4, 19). Of these potential virulence factors, TDH has been considered very important because of a striking epidemiological correlation with the Kanagawa phenomenon (KP), which is beta-hemolysis induced by TDH in a special blood agar medium (Wagatsuma agar). The KP was observed with 88 to 96% of strains from clinical specimens and with 1 to 2% of strains from nonclinical sources (24, 32). The biological activities of TDH include hemolysis of various species of erythrocytes, cytotoxicity, lethal toxicity for small experimental animals, stimulation of fluid accumulation in the rabbit ileal loop (RIL), and production of vascular permeability in rabbit skin (21, 38).

The RIL assay, which detects intestinal fluid accumulation induced by an enterotoxin, was originally developed to study the enterotoxigenicity of *Vibrio cholerae* (5). Although the capacity of TDH to induce fluid accumulation in the RIL was suggested in some studies, the association of TDH and/or the KP with enterotoxigenicity is quite unclear from the literature. At least two studies tested whole cultures of *V. parahaemolyticus* strains and found that KP-positive strains yielded positive results more frequently than KP-negative strains (33, 40). However, results suggesting that fluid accumulation is caused by a virulence mechanism in addition to TDH (41) or by a toxin(s) other than TDH (15) were also obtained. Testing of culture filtrates containing TDH provided confusing results, because a 10-fold concentration of culture filtrates was needed to induce positive reactions in the RIL (33) but a concomitant concentration of NaCl present in the growth medium caused false-positive reactions (2, 18). One study suggesting that a toxic factor resembling TDH induced positive reactions in the RIL was done with agar-grown cell lysates (2). Only very large amounts (more than 125 μg) of purified TDH were capable of stimulating fluid accumulation in the RIL, and severe destructive histopathological changes were also observed in the inoculated loops (25, 42).

The aim of the present study was to examine the enterotoxigenicity of TDH by use of an approach previously unattempted with *V. parahaemolyticus*. Using recombinant DNA techniques, we constructed a strain of *V. parahaemolyticus* that did not produce TDH and compared the isogenic mutant and the parent strain in Ussing chambers and by RIL assays. This task was complicated by the fact that KP-positive strains carry two nonidentical copies of the gene encoding TDH, designated *tdh*1 and *tdh*2. Our previous results (28, 30) showed that both gene copies contribute to the production of TDH, the *tdh*1 gene being responsible for the production of 0.5 to 9.4% of total extracellular TDH. The isogenic mutant was therefore constructed by sequential mutation of both *tdh* gene copies by in vivo recombination methods. Fresh culture filtrates of the mutant and wild-type strains were added to the luminal surface of rabbit ileal tissue mounted in Ussing chambers, and changes in short-circuit current (ΔIsc) were recorded. Ussing chambers have previously proven quite useful in investigating bacterial enterotoxins, such as the cholera enterotoxin of *V. cholerae* (8), the heat-stable enterotoxin of enterotoxigenic *Escherichia*

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coli (10), and an enterotoxin produced by enteroinvasive *E. coli* (7). For all of these enterotoxins, ΔIsc correlated with watery diarrhea and fluid accumulation in animal models. The combination of the sensitivity of the Ussing chambers and the precision of recombinant isogenic strain construction has now produced compelling evidence for the enterotoxigenicity of TDH.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. parahaemolyticus* AQ3815 (KP positive, *tdh*1 positive, *tdh*2 positive) and its isogenic mutant lacking one *tdh* gene copy, AQ3815ΔTDH1 (KP positive, *tdh*1 negative, *tdh*2 positive), were described previously (28). *E. coli* SM10 λ *pir* (23) was obtained from J. J. Mekalanos; the *pir* gene product supports the propagation of pCVD557 and other plasmids containing the R6K origin of replication. Plasmids pCVD503 (27), pCVD538 (29), and pRK2013 (11) were described previously. Plasmid pCVD557 (30) is a derivative of suicide vector pJM703.1 (23) in which the ampicillin resistance marker was replaced with a chloramphenicol resistance marker and the inactivated *tdh*2 gene, along with large flanking sequences, was inserted. Plasmid pKTN115 was constructed in this study as described below.

Construction of a TDH$^-$ isogenic mutant. A TDH-negative (TDH$^-$) isogenic mutant of AQ3815 was constructed by inactivating the *tdh*2 gene of AQ3815ΔTDH1 by a suicide vector-mediated recombination method as described elsewhere (30). In brief, pCVD557 carrying the replication origin of R6K, a chloramphenicol resistance marker, and the inactivated *tdh*2 gene, along with flanking sequences, was mobilized from *E. coli* SM10 λ *pir* into AQ3815ΔTDH1. Selection of the transconjugants on TCBS agar (Difco Laboratories, Detroit, Mich.) containing chloramphenicol (20 μg/ml) identified the strain in which pCVD557 was integrated into the *tdh*2 locus of AQ3815ΔTDH1 by homologous recombination. Subsequent growth of this strain and selection for chloramphenicol sensitivity detected the derivatives in which the integrated plasmid was removed by the second homologous recombination event. Depending on the location of the crossover, the wild-type *tdh*2 gene or the mutated *tdh*2 gene remained on the chromosome. The strain (AQ3815ΔTDH1 ΔTDH2) retaining the mutated *tdh*2 gene in the chromosome was identified by Southern blot hybridization analysis with a *tdh* gene probe.

Introduction of the wild-type *tdh*2 gene into the TDH$^-$ isogenic mutant. The 8.1-kb *Sal*I fragment of pCVD538, which contains the wild-type *tdh*2 gene and flanking chromosomal sequences, was cloned into the *Sal*I site of mobilizable plasmid vector pCVD503, carrying tetracycline and chloramphenicol resistance genes. The resulting plasmid, pKTN115, carrying a chloramphenicol resistance gene, was first transformed into *E. coli* HB101 harboring helper plasmid pRK2013. pKTN115 was then mobilized from *E. coli* HB101(pRK2013, pKTN115) into *V. parahaemolyticus* AQ3815ΔTDH1ΔTDH2 by conjugation as described previously (27).

Southern blot hybridization analysis. Total DNA and plasmid DNA extracted from test organisms were digested to completion with *Hin*dIII and analyzed by Southern blot hybridization with a *tdh* gene probe under stringent conditions as described previously (28).

Assay of extracellular TDH. The test organism was grown in LB broth (22) with shaking (200 rpm) at 37°C for various times. TDH in the culture supernatant was assayed by a sensitive enzyme-linked immunosorbent assay (ELISA) with polyclonal anti-TDH serum (bead-ELISA) as described previously (31).

Ussing chamber experiments. The test organism, grown on LB agar, was inoculated into LB broth and incubated with shaking (200 rpm) for 15 h at 37°C. For strain AQ3815 ΔTDH1ΔTDH2(pCVD538), chloramphenicol (20 μg/ml) was added to the culture medium to maintain the plasmid up to this culture stage. The culture was then transferred into fresh LB broth and incubated with shaking for 13 h at 37°C. The culture supernatant was obtained by centrifugation (12,000 × *g*, 10 min) at room temperature and filtered through an acetate membrane with a 0.45-μm pore size (Nalgene syringe filter). The filtered culture supernatant was tested in Ussing chambers either immediately or after neutralization with antisera. For the neutralization experiment, 1 ml of the filtered culture supernatant was incubated with or without 50 μl of added antiserum (polyclonal anti-TDH rabbit serum or polyclonal anti-cholera enterotoxin rabbit serum) for 30 min at 37°C. The antisera had been prepared against purified TDH (obtained from Yoshifumi Takeda) and against purified choleragen (obtained from Genevieve Losonsky).

The Ussing chamber assays were conducted as previously described (12). In brief, male New Zealand White rabbits (1.5 to 2 kg) fed ad libitum were sacrificed, and a 20-cm segment of the distal ileum was excised. The excised ileum was opened along the mesenteric border and rinsed free of intestinal contents with cold Ringer's solution (53 mM NaCl, 5 mM KCl, 30.5 mM Na_2SO_4, 30.5 mM mannitol, 1.69 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25 mM Na_2HCO_3). The serosal and muscular layers were removed, and pieces of the stripped ileum were mounted in Ussing chambers (1.12-cm^2 opening). The tissues were continuously bathed in Ringer's solution maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O_2–5% CO_2. The transepithelial electrical potential difference was measured, and the short-circuit current (Isc) was determined as described previously (9). Once the tissues reached a steady-state situation, 250 μl of the test sample (filtered culture supernatant with or without neutralization treatment) was added to the mucosal side. An equal amount of the same sample was also added to the serosal side to maintain the osmotic balance. The Isc was determined at 10-min intervals until it reached a stable value. In some experiments, theophylline (5 mM) was added at 80 min and the resulting ΔIsc were recorded. Each experiment was repeated four or five times. At the end of the experiments, the mucosal tissue was removed from the chamber and fixed in 4% paraformaldehyde–1% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4). For histological examination, the intestinal tissue was postfixed in 1% OsO_4, dehydrated with a graded ethanol series, and embedded in Epon. Sections cut at 0.5 μm were stained with toluidine blue.

RIL studies. RIL studies were conducted with 2-kg male New Zealand White rabbits. Rabbits were subjected to fasting for 48 h prior to surgery and were provided water ad libitum. An aseptic surgical technique was conducted under anesthesia induced with a mixture of ketamine (50 mg/kg), xylazine (8 mg/kg), and acepromazine (1 mg/kg) administered intramuscularly. A laparotomy was performed to externalize the intestine. Six loops were placed in the jejunum of each of two rabbits. Each loop was approximately 10 cm in length and tied at each end, with a 1-cm spacing interloop, by use of 3-0 Dexon-S sutures (Davis-Geck Inc., American Cyanamid Co., Manati, P.R.). Test strains were grown in brain heart infusion broth (Difco) with added NaCl (final

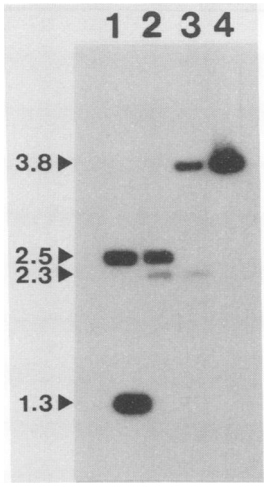

FIG. 1. Southern blot hybridization analysis of AQ3815, AQ3815ΔTDH1, and a *tdh*2-deficient derivative of AQ3815ΔTDH1 (AQ3815ΔTDH1ΔTDH2). *Hin*dIII digests of the test DNAs were analyzed by Southern blot hybridization with a *tdh* gene probe (28). Lanes: 1, total DNA of AQ3815; 2, total DNA of AQ3815ΔTDH1; 3, total DNA of AQ3815ΔTDH1ΔTDH2; 4, purified DNA of pCVD557. The sizes (in kilobases) of the probe-positive bands are indicated.

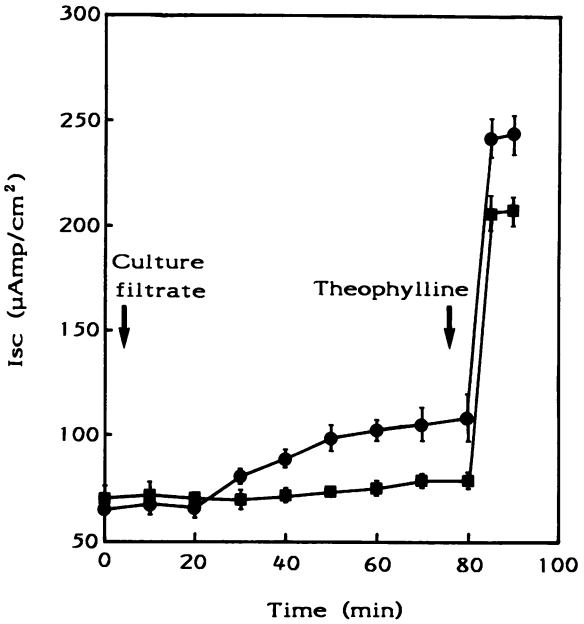

FIG. 2. Effects of culture filtrates on the Isc of rabbit ileal tissue mounted in Ussing chambers. At the indicated times, culture filtrates of AQ3815 (●) and AQ3815ΔTDH1ΔTDH2 (■) were added to the mucosal and serosal sides, and theophylline (5 mM) was added to the serosal side. Values are expressed as the mean ± standard error of the mean (n = 5).

concentration, 2%), and the concentrations of the viable cells were adjusted to ca. 10^8 cells per ml. A 1-ml inoculum was injected into the lumen of each loop in random order. Three loops were challenged with the parent strain (AQ3815) and four loops were challenged with the mutant strain (AQ3815ΔTDH1ΔTDH2) in the two rabbits. The bowel was returned to the abdominal cavity, and the incision was closed by use of 2-0 chromic gut (Davis-Geck Inc.) for interrupted sutures in the abdominal fascia and 2-0 Dermelon (Davis-Geck Inc.) for interrupted skin sutures. The rabbits were sacrificed at 18 h postinoculation with 100 mg of sodium pentobarbital per kg under ketamine (50 mg/kg) anesthesia. The fluid volume in each of the intestinal loops and the length of each of the loops were measured.

RESULTS

Construction of a TDH$^-$ isogenic mutant. For construction of an isogenic mutant of AQ3815 (*tdh*1 positive, *tdh*2 positive) that did not produce TDH, the *tdh*2 gene of AQ3815ΔTDH1, a *tdh*1-deficient isogenic mutant of AQ3815 constructed previously (28), was replaced with the inactivated *tdh*2 gene by the suicide vector-mediated recombination method described above. The final isogenic mutant was identified by Southern blot hybridization analysis with a *tdh* gene probe containing sequences flanking the deleted portions of the *tdh* genes of the mutant strains (28, 30) (Fig. 1). For both gene copies, the original inactivating mutation was constructed by the deletion of an internal restriction fragment and the addition of an antibiotic resistance gene. The *tdh*1 and *tdh*2 genes of wild-type AQ3815 are present on 1.3- and 2.5-kb *Hin*dIII fragments, respectively (28; Fig. 1, lane 1). The inactivated *tdh*1 gene in AQ3815ΔTDH1 was identified by the loss of the 1.3-kb fragment and the appearance of a 2.3-kb *Hin*dIII fragment exhibiting a weak hybridization signal with the probe (28; Fig. 1, lane 2). The inactivated *tdh*2 gene in pCVD557 was characterized by the probe-positive *Hin*dIII fragment of 3.8 kb (30; Fig. 1, lane 4). The derivative of AQ3815ΔTDH1 in which the wild-type *tdh*2 gene was replaced with the inactivated *tdh*2 gene was identified by the loss of the 2.5-kb fragment and the appearance of a 3.8-kb *Hin*dIII fragment homologous to the *tdh* gene probe. The final strain, designated AQ3815ΔTDH1 ΔTDH2, contains probe-positive *Hin*dIII fragments of 2.3 and 3.8 kb (Fig. 1, lane 3).

The genetic characterization of AQ3815ΔTDH1ΔTDH2 correlated with the phenotypic characterization; when examined by the sensitive bead-ELISA method, AQ3815 ΔTDH1ΔTDH2 did not produce extracellular TDH in LB broth after 12 or 18 h of incubation.

Introduction of the wild-type *tdh*2 gene into the TDH$^-$ isogenic mutant. To confirm that the loss of TDH production was due to the specific deletions and insertions constructed in the *tdh* structural genes, we mobilized the wild-type *tdh*2 gene cloned into a plasmid vector (pKTN115) from *E. coli* into AQ3815ΔTDH1ΔTDH2 by conjugation. The presence of pKTN115 in AQ3815ΔTDH1ΔTDH2 was confirmed by plasmid analysis (data not shown). The production of TDH was restored, and AQ3815ΔTDH1ΔTDH2(pKTN115) produced 6.4 times more TDH in the culture supernatant than AQ3815 when grown in LB broth for 18 h.

Enterotoxigenicity of TDH$^+$ and TDH$^-$ isogenic strains in Ussing chambers. The effect of culture filtrates of AQ3815 and AQ3815ΔTDH1ΔTDH2 on rabbit ileal mucosal tissue mounted in Ussing chambers was examined. Enterotoxins such as cholera toxin can affect ion flux by changing the potential difference, which is the difference in the voltages measured on the mucosal side and the serosal side of the intestinal tissue. Isc is the amount of electrical current needed to nullify the potential difference. Isc response curves are shown in Fig. 2. The culture filtrate of AQ3815 stimulated a gradual increase in Isc from 20 to 80 min after addition, whereas the culture filtrate of AQ3815ΔTDH1 ΔTDH2 induced little, if any, ΔIsc. Figure 3 shows the

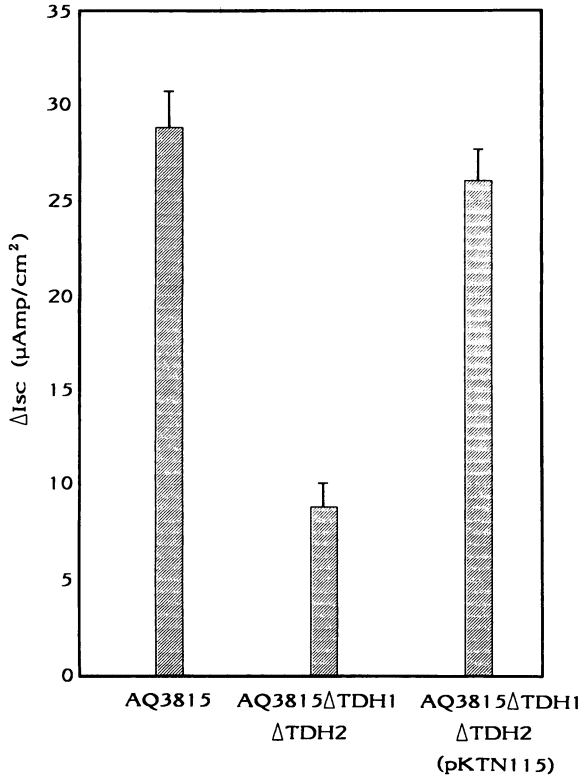

FIG. 3. Restoration of Ussing chamber activity by the addition of pKTN115, containing the cloned *tdh2* gene, to AQ3815ΔTDH1 ΔTDH2. Culture filtrates of the indicated strains were assayed. The ΔIsc values shown are the maximum ΔIsc values obtained over the course of a 90-min experiment. Values are expressed as the mean ± standard error of the mean (n = 5).

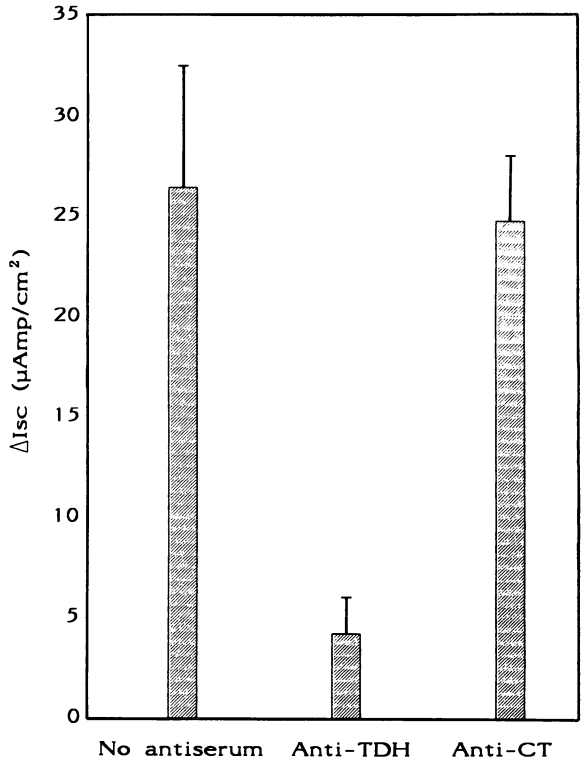

FIG. 4. Neutralization of Ussing chamber activity by the addition of anti-TDH rabbit serum to culture filtrates of *V. parahaemolyticus* AQ3815. The culture filtrates were incubated without antiserum (No antiserum), with anti-TDH serum (Anti-TDH), or with anti-cholera enterotoxin serum (Anti-CT) prior to addition to the Ussing chambers. Values are expressed as the mean ± standard error of the mean (n = 4).

maximum ΔIsc values at 90 min (27.3 ± 3.3 μA/cm^2 for AQ3815 versus 8.7 ± 1.4 μA/cm^2 for AQ3815ΔTDH1 ΔTDH2; $P < 0.02$). We had noticed, however, that AQ3815ΔTDH1ΔTDH2 grew more slowly in the test medium than AQ3815, possibly because of physiological changes associated with TDH deficiency (26). To examine the possibility that the observed ΔIsc were due to a spontaneous mutation unrelated to the *tdh2* mutation, we added the cloned wild-type *tdh2* gene on plasmid pKTN115 back to AQ3815ΔTDH1ΔTDH2. Restoration of the intact *tdh2* gene induced ΔIsc similar to those seen with wild-type strain AQ3815 (27.3 ± 3.3 versus 26.1 ± 3.0 μA/cm^2; P = not significant). Restoration of the phenotype thus fulfills the molecular Koch's postulates as defined by Falkow (6).

To further rule out the possibility that a factor(s) other than TDH contributed to the ΔIsc observed with AQ3815, we conducted neutralization experiments using anti-TDH serum. Culture filtrates of AQ3815 were incubated with anti-TDH serum, anti-cholera enterotoxin serum, or no serum. Incubation with anti-TDH serum but not with anti-cholera enterotoxin serum significantly reduced the ΔIsc response (26.4 ± 6.1 versus 4.2 ± 1.8 μA/cm^2; P = 0.05) (Fig. 4). These results confirm that TDH, alone or in combination with other factors, caused a significant increase in Isc.

The potential involvement of intracellular cyclic AMP (cAMP) in the stimulation of ΔIsc was tested by adding theophylline (an inhibitor of cAMP phosphodiesterase) to the chambers after maximum increases in Isc were induced by the culture filtrates (Fig. 2). After the addition of theophylline, equal increases in Isc were observed with tissues exposed to the culture filtrates of AQ3815 and AQ3815 ΔTDH1ΔTDH2. The equivalent responses to theophylline suggest that the ΔIsc induced by TDH may not be related to intracellular cAMP levels.

The mucosal tissues exposed to the culture filtrates of AQ3815 and AQ3815ΔTDH1ΔTDH2 in Ussing chambers were fixed and examined histologically. Tissues exposed to AQ3815 culture filtrates were indistinguishable from those exposed to filtrates of the isogenic mutant (Fig. 5), and none of the treated tissues differed from histologically normal rabbit ileal mucosal tissue (data not shown).

RIL test. Cultures of AQ3815 or AQ3815ΔTDH1ΔTDH2 containing ca. 10^8 viable cells were inoculated into ligated RILs. After 18 h of incubation, the observed fluid volume per length of ileal loop in loops inoculated with parent strain AQ3815 was 1.58 ± 0.01 ml/cm. There was no fluid accumulation in loops inoculated with AQ3815ΔTDH1ΔTDH2. The loops in both rabbits showed similar results.

DISCUSSION

A critical role of TDH in the pathogenesis of diarrhea caused by *V. parahaemolyticus* has long been postulated, but evidence to confirm this role has been inadequate and conflicting. In the present study, we constructed an isogenic strain of *V. parahaemolyticus* that lacked both *tdh* gene copies and evaluated the mutant and parent strains in Ussing

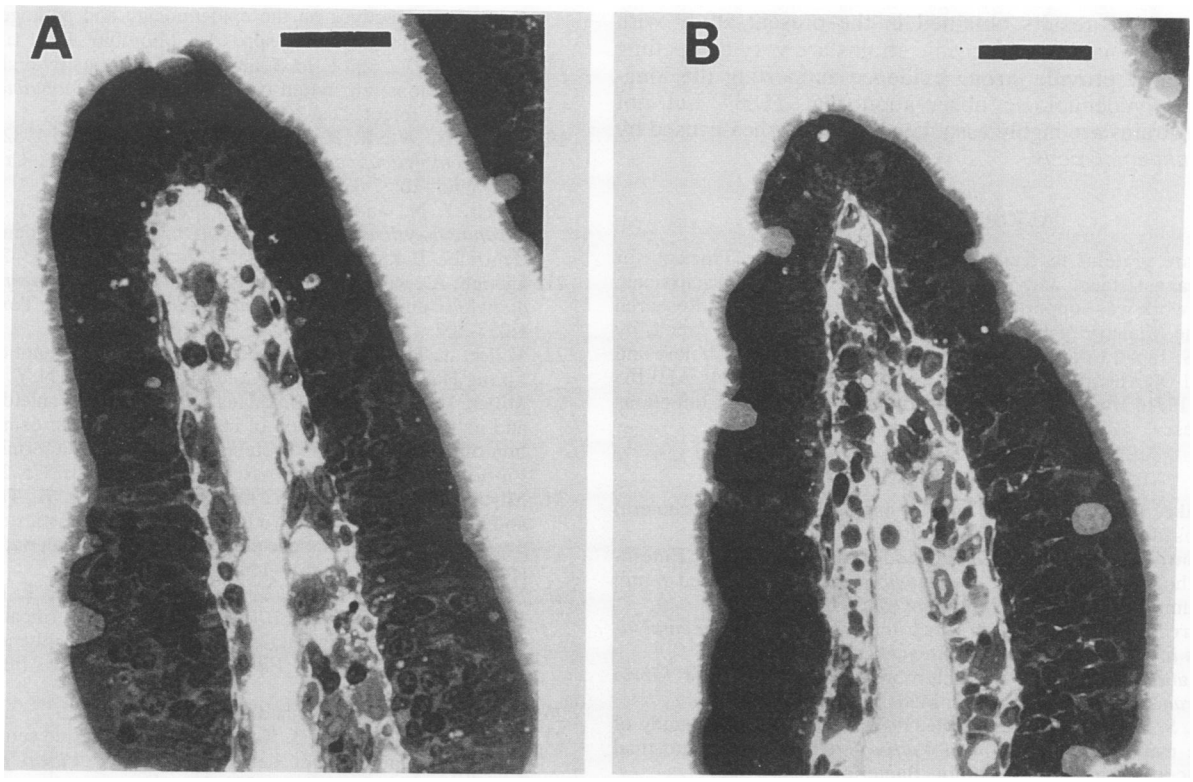

FIG. 5. Light micrographs of rabbit ileal mucosa exposed to culture filtrates of AQ3815 (A) and AQ3815ΔTDH1ΔTDH2 (B) in Ussing chambers. Bars = 10 μm.

chambers. A culture supernatant of the TDH^+ parent strain caused a striking increase in Isc, whereas that of the TDH^- mutant caused little or no change. The specificity of this activity was confirmed by the addition of the cloned, intact *tdh* gene to the mutant, with the subsequent restoration of Ussing chamber activity, and was further confirmed by neutralization experiments with anti-TDH serum. In addition, RILs inoculated with the TDH^+ parent strain showed marked fluid accumulation, but no fluid accumulation was observed in RILs inoculated with the TDH^- isogenic mutant. All of our results are consistent with the hypothesis that TDH plays a critical role in the pathogenesis of diarrhea caused by *V. parahaemolyticus*.

By inoculating bacterial cultures into RILs, one can test the potential enteropathogenicity of bacteria. However, this test is a general one that can evaluate various aspects of bacterial enteropathogenicity. Accordingly, our results cannot rule out the interpretation, based on the RIL results, that TDH has an effect on the growth rate rather than on the secretory response. The Ussing chamber assay has an advantage over the RIL assay in that a defined aspect of a bacterial enterotoxin can be examined. Although the Ussing chamber assay is a classic technique for studying bacterial enterotoxins, it has not previously been used to study changes in ion flux in intestinal epithelial cells induced by TDH. Other workers have examined the effect of TDH on myocardial tissues and erythrocytes. Seyama and coworkers (35) measured TDH-stimulated changes in the membrane resistance of the myocardial tissues of rabbits and observed that TDH increases the permeability of the myocardial membrane. Takashi et al. (37) reported that exposure to TDH in vitro induced potassium leakage from rat erythrocytes and a concomitant change in the membrane surface detectable by scanning electron microscopy. The same workers (37) also demonstrated by use of an in vivo experiment that low doses of TDH induced potassium leakage from erythrocytes but failed to induce hemolysis. The relevance of these observations to the enterotoxigenicity of TDH is not clear, but it is apparent that TDH can alter ion flux which, in the intestine, can result in secretory diarrhea. Several potential ions, e.g., Na^+, Cl^-, and HCO_3^-, and intracellular messengers, e.g., cAMP, cGMP, and Ca^{2+}, can be involved in secretory diarrhea. The results obtained with culture supernatants and measurements of Isc only are insufficient to allow us to compare the mode of action of TDH with those of other enterotoxins, such as cholera enterotoxin or *E. coli* heat-stable enterotoxin. The results obtained with theophylline suggest that cAMP is not involved in the response to TDH. In these experiments, tissues exposed to the culture filtrate of AQ3815 showed an additive effect on Isc when theophylline, an inhibitor of cAMP phosphodiesterase, was added.

The physiological concentrations of TDH used in these experiments avoided the potential problems seen with very high TDH concentrations. The concentration of TDH in the culture filtrate of AQ3815 is on the order of nanograms per milliliter (30), and it was diluted by a factor of ca. 40 in the Ussing chambers. TDH applied at this low concentration stimulated ΔIsc but did not cause damage to mucosal tissues, as determined by a histological evaluation of the intestinal tissue (Fig. 5).

Elucidation of the specific mechanisms by which TDH alters ion flux, i.e., which specific ions and intracellular messengers are involved, will require further experiments.

However, the results obtained in the present study with culture supernatants of isogenic strains are very compelling in that they provide strong evidence supporting the long-standing epidemiologic observation that TDH^+ but not TDH^- strains are highly associated with diarrhea caused by *V. parahaemolyticus*.

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